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NOVEL STRAIN OF STREPTOMYCES FOR CONTROLLING PLANT DISEASES

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A NOVEL STRAIN OF *Streptomyces* FOR CONTROLLING PLANT DISEASES

Cross-Reference to Related Applications

This application is a continuation-in-part of U.S. Application Serial No. 09/671,943 filed September 27, 2000. The contents of this application is hereby incorporated by reference into the present disclosure.

Field of the Invention

The present invention is in the field of biopesticides.

Background of the Invention

For a number of years, it has been known that various microorganisms exhibit biological activity so as to be useful to control plant diseases. Although progress has been made in the field of identifying and developing biological pesticides for controlling various plant diseases of agronomic and horticultural importance, most of the pesticides in use are still synthetic compounds. Many of these chemical fungicides are classified as carcinogens by the EPA and are toxic to wildlife and other non-target species. In addition, pathogens may develop resistance to chemical pesticides (Schwinn *et al.*, 1991).

Biological control offers an attractive alternative to synthetic chemical fungicides. Biopesticides (living organisms and the compounds naturally produced by these organisms) can be safer, more biodegradable, and less expensive to develop.

The actinomycetes, including the streptomycetes, are known producers of antifungal metabolites (Lechavalier and Waksman, 1962; Lechavalier, 1988). Several actinomycete-produced antibiotics are routinely used in an agricultural setting such as streptomycin and terramycin for fire blight control.

Streptomycetes have demonstrated both *in vitro* and *in vivo* activity against plant pathogens. Axelrood *et al.* (1996) isolated 298 actinomycetes from Douglas-fir roots. Approximately 30% of these strains demonstrated antifungal activity against Fusarium, Cylindrocarpon, and/or Pythium *in vitro*. Yuan and Crawford (1995) reported that *Streptomyces lydicus* WYEC108 showed both strong *in vitro* antifungal

activity and inhibition of Pythium root rot in pot tests with pea or cotton seed. Reddi and Rao (1971) controlled Pythium damping-off in tomatoes and Fusarium wilt of cotton with *Streptomyces ambofaciens*. Rhizoctonia root rot was controlled by *Streptomyces hygroscopicus* var. *geldanus* (Rothrock and Gottlieb, 1984). These authors reported that the control was dependent on the in situ geldanamycin concentration produced by this strain. The same authors also saw protection of soybeans from *Phytophthora megasperma* var. *sojae* by *Streptomyces herbaricolor* and *Streptomyces coeruleofuscus* (1984). Chamberlain and Crawford (1999) saw *in vitro* and *in vivo* antagonism of turfgrass fungal pathogens by *S. hygroscopicus* strain YCED9. Crawford (1996) patented the use of this strain to control plant pathogens in US patent 5, 527,526. Suh (1998) patented 2 *Streptomyces* sp. that were active against *Rhizoctonia solani* and *Phytophthora capsici*. A *Streptomyces griseoviridis* product against *Fusarium* spp. and other soil pathogens is on the market as Mycostop™.

Summary of the Invention

A novel antibiotic-producing *Streptomyces* sp. is provided that exhibits antifungal activity only on certain specific plant pathogens. Also provided is a method of treating or protecting plants from fungal infections comprising applying an effective amount of an antibiotic-producing *Streptomyces* sp. having all the identifying characteristics of NRRL Accession number B-30145. The invention also relates to fungicidal compositions comprising this novel *Streptomyces* strain and the antibiotics and metabolites produced by this strain either alone, or in combination with other chemical and biological pesticides.

The antibiotic-producing *Streptomyces* sp. can be provided as a suspension in a whole broth culture or as an antibiotic-containing supernatant obtained from a whole broth culture of an antibiotic-producing *Streptomyces* sp. Also provided is a novel butanol-soluble antibiotic that exhibits specific antifungal activity and a process for isolating the novel butanol-soluble antibiotic.

Brief Description of the Figures

Figure 1A is the analytical HPLC chromatogram of active fraction 6. (Microsorb C18, 10cm x 4.6mm, 100Å, flow rate 1mL/min, UV detection at 220nm, acetonitrile + 0.05% TFA/water + 0.05% gradient as follows: 0-30 min, 5-65%; 30-40 min, 65-100%; 40-45 min, 100%).

Figure 1B is the UV spectrum of the active peak eluting at 14.755 minutes in the chromatogram described in 1A.

Figure 2A is the analytical HPLC chromatogram of active fraction 7 under the same conditions described in 1A.

Figure 2B is the UV spectrum of the active peak eluting at 16.146 minutes in the chromatogram described in 2A.

Figure 3 is the C-8 HPLC chromatogram of the methanol eluate from the Diaion HP-20 resin step described in Method B. (HP Zorbax Eclips XDB-C8 column, 5μm, 150 x 4.6mm, flow rate 0.8 mL min, UV detection at 220 nm, chart speed 2mm/min. Solvent A, 25:5:70 acetonitrile/methanol/water. Solvent B, 65:5:30 acetonitrile/methanol/water. Gradient: 100% A at 0 minutes increased to 3% B over 20 minutes.)

Figure 4 is the ^{1}H NMR spectrum (400MHz, CD_3OD) of the semi-pure active metabolite obtained from purification method A.

Figure 5 is the ^{13}C NMR spectrum (100MHz, CD_3OD) of the semi-pure active metabolite obtained from purification method A.

Figure 6 is the LC ESI-MS (Liquid Chromatography ElectroSpray Impact – Mass Spectrum) of Peak A obtained from purification method B. (Microsorb C18, 10cm x 4.6mm, 100Å, flow rate 1mL/min, acetonitrile + 0.02% TFA/water + 0.02% gradient as follows: 0-30 min, 5-65%; 30-40 min, 65-100%; 40-45 min, 100%).

Figure 7 is the LC ESI-MS (Liquid Chromatography ElectroSpray Impact – Mass Spectrum) of Peak B obtained from purification method B. (Microsorb C18, 10cm x 4.6mm, 100Å, flow rate 1mL/min, acetonitrile + 0.02% TFA/water + 0.02% gradient as follows: 0-30 min, 5-65%; 30-40 min, 65-100%; 40-45 min, 100%).

Detailed Description

The present invention provides a novel strain of *Streptomyces sp.* or mutants thereof with antifungal activity only on specific plant pathogens such as *Alternaria*, *Phytophthora*, *Botrytis*, *Rhizoctonia* and *Sclerotinia*. This novel strain was deposited with the NRRL on July 20, 1999 under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure under Accession No. B-30145. The invention also includes methods of preventing and treating fungal diseases in plants using such bacterial strains or antibiotic-containing supernatants or pure antibiotics obtained from such bacterial strains. The invention also includes a butanol soluble antifungal antibiotic with a molecular weight of less than 10,000 daltons, with stability to base and to heat treatment of 1 hour at 80°C and lability to acid treatment.

Definitions

The singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

The term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and agriculturally acceptable carriers. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for applying the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

As used herein, "biological control" is defined as control of a pathogen or insect by the use of a second organism. Known mechanisms of biological control include enteric bacteria that control root rot by out-competing fungi for space on the surface of the root. Bacterial toxins, such as antibiotics, have been used to control

pathogens. The toxin can be isolated and applied directly to the plant or the bacterial species may be administered so it produces the toxin *in situ*.

The term "fungus" or "fungi" includes a wide variety of nucleated spore-bearing organisms that are devoid of chlorophyll. Examples of fungi include yeasts, molds, mildews, rusts, and mushrooms.

The term "bacteria" includes any prokaryotic organism that does not have a distinct nucleus.

"Pesticidal" means the ability of a substance to increase mortality or inhibit the growth rate of plant pests.

"Fungicidal" means the ability of a substance to increase mortality or inhibit the growth rate of fungi.

"Antibiotic" includes any substance that is able to kill or inhibit a microorganism. Antibiotics may be produced by a microorganism or by a synthetic process or semisynthetic process. The term, therefore, includes a substance that inhibits or kills fungi for example, cycloheximide or nystatin.

"Antifungal" includes any substance that is able to kill or inhibit the growth of fungi.

The term "culturing" refers to the propagation of organisms on or in media of various kinds. "Whole broth culture" refers to a liquid culture containing both cells and media. "Supernatant" refers to the liquid broth remaining when cells grown in broth are removed by centrifugation, filtration, sedimentation, or other means well known in the art.

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations. In terms of treatment and protection, an "effective amount" is that amount sufficient to ameliorate, stabilize, reverse, slow or delay progression of the fungal or bacterial disease states.

"Positive control" means a compound known to have pesticidal activity. "Positive controls" include, but are not limited to commercially available chemical pesticides. The term "negative control" means a compound known not to have pesticidal activity. Examples of negative controls are water or ethyl acetate.

The term "solvent" includes any liquid that holds another substance in solution. "Solvent extractable" refers to any compound that dissolves in a solvent and which then may be isolated from the solvent. Examples of solvents include, but are not limited to, organic solvents like ethyl acetate.

The term "metabolite" refers to any compound, substance or byproduct of a fermentation of a microorganism that has pesticidal activity. Antibiotic as defined above is a metabolite specifically active against a microorganism.

The term "mutant" refers to a variant of the parental strain as well as methods for obtaining a mutant or variant in which the pesticidal activity is greater than that expressed by the parental strain. The "parent strain" is defined herein as the original *Streptomyces* strain before mutagenesis. To obtain such mutants the parental strain may be treated with a chemical such as N-methyl-N'-nitro-N-nitrosoguanidine, ethylmethanesulfone, or by irradiation using gamma, x-ray, or UV-irradiation, or by other means well known to those practiced in the art.

A "composition" is intended to mean a combination of active agent and another compound, carrier or composition, inert (for example, a detectable agent or label or liquid carrier) or active, such as an adjuvant. Examples of agricultural carriers are provided below.

We describe a novel antibiotic-producing strain of *Streptomyces* sp. NRRL No. B-30145 and mutants thereof that have antifungal activity only on specific plant pathogens. Also provided is a supernatant isolated from the culture as well as a composition comprising the culture. In a further aspect, the compositions further comprise at least one chemical or biological pesticide.

A metabolite produced by the *Streptomyces* sp. strain is also provided by this invention. The metabolite exhibits activity against plant pathogenic fungi and is heat and base stable, is acid labile and has a molecular weight of less than 10,000 daltons. By way of example, the metabolite may have a molecular weight [M + H^+] between about 925 to between about 865.

The one or more metabolites produced by the *Streptomyces* sp. strain exhibit UV absorption between about 215 nm and 220 nm. The metabolite may be comprised of a variety of molecules including, but not limited to, propargyl alcohol segments

[C=C-CH(OH)], oxygenated methine carbons (X-CH-Y) or a sugar moiety. By way of example, the metabolite may comprise at least two propargyl segments, several oxygenated methine carbons (by way of example, e.g., 5 to 10) and /or a sugar moiety. Alternatively, the one or more metabolites produced by the *Streptomyces* sp. strain may share the same carbon skeleton and differ in degree of oxygenation.

The present invention also provides antifungal compositions comprising a metabolite produced by *Streptomyces* and isolated according to a method comprising:

(a) loading a whole broth culture of *Streptomyces* sp. strain NRRL No. B-30145 or mutants thereof having all the identifying characteristics of NRRL No. B-30145 onto a non-ionic absorbent polymeric resin;

(b) eluting the metabolite with an alcohol;

(c) screening the eluate of step (b) with a bioassay for fractions of the eluate exhibiting antifungal activity;

(d) loading the fractions of the eluate exhibiting antifungal activity of step (c) on a HPLC column; and

(e) eluting the metabolite with an organic solvent.

The method may further comprise washing the resin with water prior to step (b) and screening the eluate of step (e) with a bioassay to select the fractions exhibiting antifungal activity.

The whole broth culture of step (a) may be freeze dried and re-suspended with an aqueous solution (e.g., water) prior to adding to the non-ionic absorbent polymeric resin. In a preferred embodiment the whole broth culture added to the resin is a homogenized cell-free whole broth culture. Examples of non-ionic absorbent polymeric resin that may be used include, but are not limited to, Supelco Sepabead SP-207 or Supelco Diaon HP-20.

The eluent used to remove the metabolite in step (b) may be an alcohol or a gradient of aqueous alcohol. By way of example, methanol or a gradient of aqueous methanol may be used as the eluent (e.g., Example 6).

The bioassay of step (c) may be any assay which evaluates antifungal activity. Examples of such bioassays include but are not limited to, the agar diffusion assay or

slide germination assay. For example, the bioassay may be a germination assay with *Monilinia fructicola* and/or *Alternaria brassicicola.*

Examples of an HPLC column that may be used in step (d) include, but are not limited to, C-18 or C-8. Examples of the organic solvent that may be used to remove the metabolite from the HPLC column include, but are not limited to, an acetonitrile – water gradient (e.g., Example 6).

The metabolite can also be formulated as a composition, with a carrier or alternatively, with at least one chemical or biological pesticide.

In order to achieve good dispersion and adhesion of compositions within the present invention, it may be advantageous to formulate the whole broth culture, supernatant and/or metabolite/antibiotic with components that aid dispersion and adhesion. Suitable formulations will be known to those skilled in the art (wettable powders, granules and the like, or can be microencapsulated in a suitable medium and the like, liquids such as aqueous flowables and aqueous suspensions, and emulsifiable concentrates). Other suitable formulations will be known to those skilled in the art.

The strain, culture, supernatant and isolated metabolite are useful to protect or treat plants, fruit, and roots from fungal infections by applying an effective amount of the active formulation to the plant, fruit or root. The formulations are particularly suited to treat or prevent infections caused by a fungus selected from the group consisting of *Alternaria solani*, *Botrytis cinerea, Rhizoctonia* sp., *Sclerotinia* sp., and *Phytophthora* sp.

All patents and publications cited herein are incorporated by reference. Full bibliographic citations for these may be found at the end of the specification, immediately preceding the claims.

The following examples are provided to illustrate the invention. These examples are not to be construed as limiting.

EXAMPLES

Example 1

Characterization of Strain NRRL No. B-30145

NRRL No. B-30145 was identified based on 16S rRNA sequencing. The protocol used to generate the 16S rRNA gene data sequence (Acculab Customer Handbook v. 1.0) is described as follows.

The 16S rRNA gene is PCR amplified from genomic DNA isolated from bacterial colonies. Primers used for the amplification correspond to *E. coli* positions 005 and 531. Amplification products are purified from excess primers and dNTPs using Microcon 100 (Amicon) molecular weight cut-off membranes and checked for quality and quantity by running a portion of the products on an agarose gel.

Cycle sequencing of the 16S rRNA amplification products is carried out using AmpliTaq FS DNA polymerase and dRhodamine dye terminators. Excess dye-labeled terminators were removed from the sequencing reactions using a Sephadex G-50 spin column. The products are collected by centrifugation, dried under vacuum and frozen at –20°C until ready to load. Samples are re-suspended in a solution of formamide/blue dextran/EDTA and denatured prior to loading. The samples are electrophoresed on an ABI Prism 377 DNA Sequencer. Data are analyzed using PE/Applied Biosystem's DNA editing and assembly software. Once obtained, sequences are compared against PE/Applied Biosystem's MicroSeq™ database using MicroSeq™ sequence analysis software. Sequences are also compared to the GenBank and Ribosomal Database Project (RDP).

The result of the 16S rRNA sequencing identified NRRL No. B-30145 as a *Streptomyces* sp. This strain may belong to the species *S. mashuensis* (formerly *Streptoverticillium mashuense*) or a related species, as suggested by the sequencing results. The best match was *Streptomyces mashuensis* with a 98% match score.

Example 2

Activity of NRRL No. B-30145 against plant pathogens in *in-vitro* culture (zone assay).

NRRL No. B-30145 was tested against an array of different plant pathogens utilizing two different *in-vitro* assays. The agar diffusion (zone) assay consists of applying either plant pathogen spores over the surface of an agar medium to create an entire lawn of growth or utilizing a mycelial agar plug placed in the center of the petri dish that will grow and colonize the agar. Circular wells approximately 7.0mm in diameter are removed from the agar using a pipette attached to a vacuum pump. Fermentation samples of NRRL No. B-30145 are added to each well along with known standards and water checks. Plates are incubated for three to four days under environmental conditions conducive for each pathogen. Results consist of a zone of no pathogen growth around the well or a greatly reduced amount of pathogen growth around the well or no affect. The size and type of zone is recorded for each sample. Results for NRRL No. B-30145 in agar diffusion assays are presented in Table 1. Results within agar diffusion were variable; diffusion through agar may be inhibited.

Table 1: Activity of NRRL No. B-30145 against selected plant pathogens in the agar diffusion (zone) assay.

Alternaria brassicicola	No Zone / Weak Activity
Botrytis cinerea	Weak Activity
Monilinia fructicola	No Zone
Phytophthora capsici	Moderate activity
Pythium sp.	Weak Activity
Colletotrichum acutatum	No Zone
Rhizoctonia solani	No Zone
Sclerotinia sclerotiorum	No Zone

The second type of *in-vitro* assay performed to test the pathogen spectrum of NRRL No. B-30145 was the slide germination assay. Fermentation samples of NRRL No. B-30145 at various dilutions were added to glass depression slides (25mm x

75mm with 18mm diameter depression 1.75mm deep) and an equal volume of pathogen spores were mixed with the sample. Slides were incubated on moistened paper towels in sealed plastic boxes at room temperature overnight. Results are determined by observing the fermentation sample / spore suspension sample using a compound microscope at 100X. Typical results consist of lack of germination of the pathogen propagule or greatly reduced germination and/or growth. In addition, various types of malformations of the initial growth from the pathogen spores may occur. The spectrum of activity of NRRL No. B-30145 is presented in Table 2. Complete inhibition of spore germination occurred at low concentrations of fermentation samples.

Table 2: Activity of NRRL No. B-30145 against selected plant pathogens in the slide germination assay.

Alternaria brassicicola	No Germination
Alternaria dauci	No Germination
Botrytis cinerea	No Germination
Monilinia fructicola	No Germination

Example 3

Activity of NRRL against plant pathogens in plant bioassay tests.

Activity of NRRL No. B-30145 was tested against tomato late blight (*Phytophthora infestans*), tomato early blight (*Alternaria solani*), gray mold (*Botrytis cinerea*), turf brown patch (*Rhizoctonia* sp.), and peanut southern blight (*Sclerotinia minor*). All tests were conducted under controlled environment in the laboratory with plant material grown from seed under typical commercial greenhouse conditions.

Tomato Late Blight – *Phytophthora infestans*

The pathogen is grown on rye agar in standard petri dishes at 16°C in the dark. Sporangia are collected by flooding the plate with water and scraping the mycelium to dislodge the sporangia. The sporangial suspension is passed through cheesecloth, quantified and adjusted to 1.0×10^{-4}. Tomato seedlings at the 3rd to 5th leaf stage are

sprayed to run-off with the fermentation sample of NRRL No. B-30145 using an artist airbrush at 40psi. Treated seedlings are allowed to air dry at room temperature for at least two hours then inoculated with the sporangial suspension by lightly spraying the upper surfaces of the tomato seedlings using a hand held sprayer. Inoculated seedlings are placed in solid bottom flats filled with water and then are covered with a plastic dome to maintain leaf wetness. Flats are incubated at 20°C with a 14-hr photoperiod for four days continuously covered by the plastic domes. Seedlings are then rated based on a disease rating scale from 0 – 5 with 0 equaling no symptoms, and 5 equaling 75% or more of the foliage colonized by the pathogen. A typical example of a late blight test is presented in Table 3.

Table 3: Results of NRRL No. B-30145 treated tomato seedlings against the late blight pathogen *Phytophthora infestans*.

Treatment	Ave.D.I.	Replications 1 - 4			
Sample 990702	1.1	1.0	0.5	2.0	1.0
Sample 990709	1.1	1.0	2.0	1.0	0.5
Sample 990825	1.3	1.5	1.0	1.5	1.0
Sample 990913	1.0	1.0	1.0	1.5	0.5
Quadris 30ppm	0.1	0	0.5	0	0
Water Check	4.3	4.0	4.0	5.0	4.0

Samples are different fermentations of NRRL No. B-30145.
D.I. is Disease Index.

Tomato Early Blight – *Alternaria solani*

The pathogen is first grown on commercial Difco potato dextrose agar (PDA) at 22-25°C under 14-hour lights until the entire plate is covered. The fungus and the agar medium is then cut into small squares approximately 10mm square and placed fungus side up on a specialized sporulation medium (S-Medium: 20g sucrose, 20g calcium carbonate, 20g Bacto-agar per liter). The S-Media plates are flooded with a thin layer of water and incubated 2-3 days at 22 – 25°C under 14-hour lights until full

sporulation of the pathogen occurs. Plates are then flooded with water and the agar squares are scraped from the plate. The suspension is passed through cheesecloth and the spores are quantified and adjusted to $1.0X10^5$. Tomato seedlings at the 3rd to 5th leaf stage are then sprayed until run-off using an artist airbrush as described previously. Treated seedlings are allowed to dry and then inoculated with the spore suspension. Seedlings are placed in flats and covered as described previously and incubated at 25°C with a 14-hour photoperiod. Seedlings are rated based on a scale of 0 – 5 as previously described. Results from a typical test are presented in Table 4.

Table 4: Activity of NRRL No. B-30145 against the early blight pathogen *Alternaria solani.*

Treatments	Ave D.I.	Replications			
Test-1					
Sample 990216	1.0	2.0	0.5	0.5	
Quadris 60 ppm	1.8	1.5	2.5	1.5	
Water Check	4.0	3.0	4.0	5.0	
Test-2					
Sample 990216	1.1	1.5	1.0	1.0	1.0
Water Check	4.5	5.0	4.0	4.0	5.0

D.I. is Disease Index.

Pepper Gray Mold – *Botrytis cinerea*

The pathogen is grown on standard PDA under a 14-hour photoperiod at 22°C until the fungal growth has completely covered the plate (7-9 days). Spores are collected by flooding the plate with water and then gently scraping with a spatula to dislodge the spores. The spore suspension is passed through cheesecloth and quantified and adjusted to $1.5X10^6$. Pepper seedlings are grown until the 4th to 6th true leaf stage and fermentation samples are sprayed on the upper leaf surfaces using an artist airbrush as described previously. Treated seedlings are inoculated, placed in flats and covered with plastic domes. Flats are placed at 20°C under continuous

darkness for 2.5 days. Seedlings are rated on a 0 – 5 scale as described previously. Table 5 depicts results from two typical tests.

Table 5: Activity of NRRL No. B-30145 against *Botrytis cinerea*

Treatment	Ave D.I.	Replications			
Test -1					
Sample 990216	1.4	1.5	1.5	1.5	1.0
Break 20ppm	0.1	0	0	0.5	0
Water Check	4.0	4.0	4.0	3.0	5.0
Test-2					
Sample 990216	1.9	1.5	2.0	2.0	2.0
Break 20ppm	0.8	0	1.5	1.0	0.5
Water Check	4.5	4.0	5.0	5.0	4.0

D.I. is Disease Index.

Turf brown patch-*Rhizoctonia* sp.

Two ml of fermentation sample was added to each cell of a 6-cell pot of one-month old turf seedlings (Bentgrass). A 4 mm mycelial plug of a 2-3 day old culture of *Rhizoctonia* sp. was placed under the soil surface. Each treatment was replicated 6 times. Inoculted pots were placed in plastic flats and covered with a plastic dome. The flats were placed on a light rack (16Hr/day) and incubated at room temperature. Disease severity was evaluated after 5-6 days incubation and compared with the water treated control. The results indicated that NRRL No. B-30145 has a suppressive activity against Rhizoctonia (Table 6).

Table 6. The efficacy of NRRL No. B-30145 on turf disease caused by *Rhizoctinia* sp.

Treatment	Dilution Factor	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6
30145	1x	+*	+	+	+	+	+
30145	½ x	+	+	+	+	+	+
Water		+++	+++	+++	+++	+++	+++

* "+" = light symptoms, "+++"=severe symptoms

Peanut southern blight-*Sclerotinia minor*

Peanut seedlings at the first 2-leaf stage were treated with NRRL No. B-30145 and a 4-mm mycelial plug is placed on the base of each stem after the treated plants dried. Inoculated plants were incubated in a dew chamber for 2 days before being placed in a plastic flat sealed with a cover dome. The flat was incubated on a light rack (16Hr/day) at room temperature for 10 days. Disease severity was assessed by comparing the treated with the water control. The results (Table 7) indicated NRRL No. B-30145 whole broth at 1x has some controlling activity against *Sclerotinia minor.*

Table 7. The efficacy of NRRL No. B-30145 on peanut Sclerotinia blight.

Treatment	Dilution Factor	Rep 1	Rep 2	Rep 3
30145	1x	+/-	0	+/-
30145	½ x	++	++	+
30145	¼ x	0	++	++
Water		+++	+++	+++

*"+/- " indicates strong suppression, 0 indicates no infection, "+" = light symptoms, "+++"=severe symptoms,.

Example 5

Antifungal metabolite produced by NRRL No. B-30145.

The whole broth of NRRL No. B30145 was partitioned into ethyl acetate, butanol and aqueous fractions. Each fraction was tested against *Alternaria brassicicola* in a spore germination assay. The *Alternaria* spores were germinated in the presence of each sample in depression microscope slides containing 40 µl of sample and 20 µl of pathogen spores. Approximately 16 hours later the spores are

observed under a microscope to see if they have germinated. No germination (score of 0) compared to the water control (100% germination and growth=score of 5) indicates activity of the sample being tested. Results of the *Alternaria* germination assay with different NRRL No. B-30145 fractions are shown below (score on a 0 to 5 rating as above).

Fraction	**Score**		
	Rep 1	Rep 2	Rep3
Ethyl acetate	3	nd	4
n-butanol	0	0.2	1
Aqueous	0	5	5
Whole broth	0	0.2	0
Water Check	5	5	5

The metabolite is clearly in the butanol soluble fraction and is not readily extractable in ethyl acetate. Other characteristics of the metabolite were determined. The molecule was shown to pass through a 10,000 molecular weight cut off filter indicating the metabolite is smaller than 10,000 daltons. The activity was not lost when treated with base or upon heating to 80 degrees C for one hour. The activity was lost when treated with acid (the score against *Alternaria* increased from 0 to 5).

Fractionation of the butanol extract on octadecylsilane bonded to silica gel (ODS) flash chromotography using an acetonitrile (ACN)/water gradient with 0.01 % trifluoroacetic acid (TFA) yielded an active fraction eluting with 50% acetonitrile/water with 0.01 % TFA. Fractions were tested in an *Alternaria* germination assay for activity (0-5 rating scale).

Fraction	Score
ODS 10% ACN	4
ODS 20% ACN	5
ODS 50% ACN	0.5
ODS 100% ACN	5
Water Check	5

Further purification by ODS HPLC yielded 2 active components (Fraction 6 and 7) from an isocratic elution with 31% acetonitrile in water with 0.02 % TFA).

Fraction	Score
HPLC Fr.1	5
HPLC Fr.2	5
HPLC Fr.3-5	4
HPLC Fr.6	3
HPLC Fr.7	2
HPLC Fr.8	5
HPLC Fr.9	5
Water Check	5

The HPLC chromatogram of the active 50% acetonitrile/water with 0.01 % TFA flash chromotography fraction and the HPLC chromatograms of the active fractions 6 and 7, including UV spectra of the active principles, are shown in Figures 1 and 2.

NRRL No. B-30145 most closely matched *Streptomyces mashuensis* by 16S RNA sequencing. Unlike the antibacterial metabolites typically associated with *S. mashuensis*, the fungicidal activity of NRRL No. B-30145 was extractable with butanol. *S. mashuensis* is known to produce streptomycin, which is a water-soluble antibacterial compound. Another antibiotic produced by *S. mashuensis,* monazomycin (Akasaki *et al.* 1963), does not display a shoulder at 215 –220 nm as does the fungicidal active fractions of NRRL No. B-30145.

Antifungal compounds have also been found in the closely related and possibly synonymous species *Streptomyces griseocarneum* (American Type Culture Collection). These include porfiromycin (Claridge *et al.*, 1986), a purple compound whose corresponding UV spectrum is not seen in the active fraction of NRRL No. B-30145 and the *Heptaenes trichomycin* (Komori and Morimoto, 1989) and *griseocarnin* (Campos *et al.*, 1974), whose corresponding UV spectra are also not present in the active fraction. The fungicidal active is also not neutramycin, which is extractable with ethyl acetate (Mitscher and Kunstmann, 1969).

Example 6

Additional Methods for further purification of the Antifungal metabolite of NRRL No. B-30145

Method A

The freeze-dried whole broth culture was re-suspended in water (2. 0 L) and loaded onto a column containing a non-ionic polymeric resin (Supelco Sepabead SP-207; 26 x 3.0 cm) equilibrated in water. The column was washed with water (200 mL) and then with a gradient of aqueous methanol as follows: (1) 20:80 methanol/water (200 mL), (2) 40:60 methanol/water (200 mL), (3) 60:40 methanol/water (200 mL), 80/20 methanol/water (200 mL), and (5) methanol (200 mL).

Bioassay results (germination assay with *Monilinia fructicola* and/or *Alternaria brassicicola*) indicated that all fractions were active. Each fraction was individually fractionated on octadecylsilane-bonded silica gel (ODS) HPLC using an acetonitrile/methanol/water (TOSOHASS ODS-80TS; 10 μm, 21.5 x 30 cm. Solvent system: solvent A: acetonitrile/methanol/water 25:5:65, solvent B: acetonitrile/methanol/water 65:5:30. Gradient: start at 0 min with solvent A and hold for 25 min. Then increase solvent B to 35% over 50 min. Flow = 6.0 mL/min). All fractions yielded approximately the same HPLC profile with the activity located at two regions: peak A (t ~ 55-63 min) and peak B (t ~ 65-70 min).

Peak B was further fractionated on another reversed-phase HPLC column (Phenomenex Luna Phenyl-Hexyl; 5 μm, 250 x 10 mm. Solvent system: solvent A:

acetonitrile/methanol/water 25:5:65, solvent B: acetonitrile/methanol/water 65:5:30. Gradient: start at 0 min with solvent A and hold for 15 min. Then increase solvent B to 25% over 25 min. Flow = 2.0 mL/min). One major component was isolated; however, analytical HPLC analysis indicated a high-UV absorbing contaminant that co-eluted with active metabolite. Therefore, an alternative purification method was employed (method B).

Method B

Alternatively, the homogenized cell-free whole broth culture is passed through non-ionic polymeric resin (Supelco Diaion HP-20), washed with water, and then methanol. The methanol eluate is further separated by reversed-phase HPLC (HP Zorbax Eclipse XDB-C8; 5 μm, 150 x 4.6 mm. Solvent system: solvent A: acetonitrile/methanol/water 25:5:65, solvent B: acetonitrile/methanol/water 65:5:30. Gradient: start at 0 min with solvent A and increase solvent B to 3% in 20 min. Flow = 0.8 mL/min) to afford the same active peaks observed in method A (peaks A and B) and confirmed by analytical HPLC using UV and MS detection. An HPLC trace is shown in Figure 3.

Characteristics of active metabolites of NRRL No. B-30145

The impure fraction obtained from method A provided some initial information about the nature of the active metabolite. LC MS indicated a molecular weight $[M + H^+]$ = 892.6 and the UV spectrum displays a shoulder at 215-220 nm. 1D and 2D NMR suggests at least 2 propargyl alcohol segments [C=C-CH(OH)], several oxygenated methine carbons (X-CH-Y), and a possible sugar moiety. ^{1}H and ^{13}C NMR are shown in Figures 4 and 5 respectively.

Even though method B has not provided sufficient quantities for NMR analysis, this method yielded cleaner peaks in sufficient amounts for analysis by HPLC (octyl bonded silica gel) using UV and MS detection methods. Two major peaks (peak A and B) were obtained that matched the same compounds identified as the active metabolites using method A (see Figure 3). The UV spectra of all compounds presented a shoulder at 215-220 nm. LC MS of peak A indicated the presence of at least three (3) compounds with the following molecular weights $[M + H^+]$ = 866.5, 882.5, and 898.4 (see Figure 6). Similarly, peak B showed at least three (3)

compounds with molecular weights $[M + H^{+}] = 892.5$, 908.5, and 924.5 (see Figure 7).

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications will be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

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